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The α-Phosphoglucomutase of *Lactococcus lactis* Is Unrelated to the α-D-Phosphohexomutase Superfamily and Is Encoded by the Essential Gene *pgmH*[*][S]

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α-Phosphoglucomutase (α-PGM) plays an important role in carbohydrate metabolism by catalyzing the reversible conversion of α-glucose 1-phosphate to glucose 6-phosphate. Isolation of α-PGM activity from cell extracts of *Lactococcus lactis* strain MG1363 led to the conclusion that this activity is encoded by *yfgH*, herein renamed *pgmH*. Its gene product has no sequence homology to proteins in the α-D-phosphohexomutase superfamily and is instead related to the eukaryotic phosphomannomutases within the haloacid dehalogenase superfamily. In contrast to known bacterial α-PGMs, this 28-kDa enzyme is highly specific for α-glucose 1-phosphate and glucose 6-phosphate and showed no activity for mannose phosphate. To elucidate the function of *pgmH*, the metabolism of glucose and galactose was characterized in mutants overproducing or with a deficiency of α-PGM activity. Overproduction of α-PGM led to increased glycolytic flux and growth rate on galactose. Despite several attempts, we failed to obtain a deletion mutant of *pgmH*. The essentiality of this gene was proven by using a conditional knock-out strain in which a native copy of the gene was provided in *trans* under the control of the nisin promoter. Growth of this strain was severely impaired when α-PGM activity was below the control level. We show that the novel *L. lactis* α-PGM is the only enzyme that mediates the interconversion of α-glucose 1-phosphate to glucose 6-phosphate and is essential for growth.

Phosphoglucomutase (PGM[3]; EC 5.4.2.2) is widespread in living organisms from bacteria to humans (1). It plays various roles in carbohydrate metabolism by catalyzing the reversible conversion of α-Glc-1-P to Glc-6-P. In higher organisms, its major function is mediating the mobilization of sugar moieties from energy reserves. Also, α-PGM activity is essential for the synthesis of UDP-glucose, a sugar donor for the production of glucose-containing polysaccharides. Therefore, PGM is a crucial link between catabolic and anabolic processes.

Lactococcus lactis is used worldwide in the industrial manufacture of fermented milk products. The organism converts sugars primarily into lactic acid, thus providing an efficient means of food conservation. In *L. lactis*, PGM is assumed to be essential for the utilization of galactose via the Leloir pathway (2) and for the synthesis of cell wall polysaccharides and exopolysaccharides (3, 4). In a number of Gram-positive bacteria, *pgm* mutants show altered cell wall morphology and altered polysaccharide production as well as growth defects on glucose (5–7). Despite the wealth of knowledge on sugar metabolism of *L. lactis* (8), genes coding for α-PGM have not been identified in this organism.

More than 1 decade ago, the presence of two distinct PGM activities in *L. lactis* ssp. *lactis* with specificity for α- and β-anomers of phosphoglucose was reported (9). A 28-kDa protein (designated β-PGM) was shown to catalyze the reversible conversion of β-Glc-1-P to Glc-6-P. *L. lactis* β-PGM, which belongs to the haloacid dehalogenase (HAD) superfamily (10–12), is a catabolic enzyme in the pathway for maltose and trehalose degradation encoded by a gene (*pgmB*) in the trehalose operon (13–15). A larger protein (~65 kDa) showing affinity for the α-anomer of Glc-1-P was partially purified (9). The α-specificity and protein size, which matched the analogous parameters of bacterial α-PGMs, led to the conclusion that it was the lactococcal α-PGM. Thus far, all α-PGMs described belong to the α-D-phosphohexomutase superfamily of proteins (1, 16). Intriguingly, a BLASTp search of the available *L. lactis* genome sequences (14)[4] using members of the α-D-phosphohexomutase superfamily as query or a domain search using the highly conserved regions of proteins in this family retrieved only FemD, a protein tentatively annotated as a phosphoglucosamine mutase.

In this work, we report the identification, purification, expression, and characterization of *L. lactis* α-PGM and its

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[3] The abbreviations used are: PGM, phosphoglucomutase; HAD, haloacid dehalogenase; TEA, triethanolamine; BisTris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane; Fru-1,6-P_2, fructose 1,6-bisphosphate; PMMs, phosphomannomutases.

[4] U. Wegmann, M. O'Connell-Motherway, A. Zomer, G. Buist, C. Shearman, C. Canchaya, M. Ventura, A. Goesmann, M. J. Gasson, O. P. Kuipers, D. van Sinderen and J. Kok, unpublished data.

TABLE 1
Strains and plasmids used in this study
Cm^r, chloramphenicol-resistant; Em^r, erythromycin-resistant.

	Description	Ref.
Strain		
MG1363	Plasmid-free and prophage-cured derivative of NCDO712	23
NZ9000	MG1363 derivative carrying *pepN*::*nisRK*	17
LL302	*repA*$^+$ MG1363 carrying single copy of pWV01 *repA* in *pepX*	25
NZ9000::pORI280Δ*pgmH*	NZ9000 derivative containing pORI280Δ*pgmH* integrated in chromosome in *pgmH*	This work
NZ9000Δ*pgmH*(*pgmH*$^+$)	NZ9000 derivative containing 711-bp deletion in *pgmH* and carrying pNZ8048-*pgmH*	This work
Plasmid		
pNZ8048	Cm^r; inducible expression vector carrying P_{nisA}	17
pNZ8048-*pgmH*	Cm^r; derivative of pNZ8048 carrying copy of *pgmH*	This work
pORI280	Em^r; *ori*$^+$ *repA*$^-$ derivative of pWV01; *lacZ* expressed constitutively via promoter P32	24
pORI280Δ*pgmH*	Em^r; derivative of pORI280 specific for integration in *L. lactis pgmH* gene	This work
pORI13	Em^r; integration vector; *ori*$^+$ *repA*$^-$ derivative of pWV01; promoterless *lacZ*	27
pORI13-*pgmH*′	Em^r; derivative of pORI13 specific for integration in *L. lactis pgmH* gene	This work
pVE6007	Cm^r; temperature-sensitive derivative of pWV01	26
pNZ8048-*femD*	Cm^r; derivative of pNZ8048 carrying copy of *femD*	This work
pNZ8048-*galE*	Cm^r; derivative of pNZ8048 carrying copy of *galE*	This work

encoding gene *pgmH* (previously know as *yfgH*) and show that this activity is essential for growth. To our knowledge, lactococcal α-PGM is the first member of the HAD superfamily of proteins with strict specificity for α-Glc-1-P.

EXPERIMENTAL PROCEDURES

Microbial Strains and Growth Conditions—The strains and plasmids used throughout this study are listed in Table 1. For molecular biology procedures, *L. lactis* strains were cultivated as batch cultures (flasks) without aeration in M17 medium (Difco) containing 0.5% (w/v) glucose at 30 or 38 °C. For physiological studies, *L. lactis* NZ9000 (17) derivatives NZ9000(pNZ8048) (control strain), NZ9000(pNZ8048-*pgmH*) (hereafter designated NZ9000(*pgmH*$^+$)), and NZ9000Δ*pgmH*-(*pgmH*$^+$) were grown in chemically defined medium at 30 °C under anaerobic conditions at pH 6.5 as described previously (18). Glucose or galactose was added to a final concentration of 1% (w/v). Plasmid selection was achieved by the addition of chloramphenicol (5 mg/liter) or erythromycin (5 mg/liter). For overproduction of α-PGM, nisin (1 μg/liter) was added at A_{600} = 0.5. For studies in which the nisin-inducible conditional mutant NZ9000Δ*pgmH*(*pgmH*$^+$) was used, cells were grown in M17 medium containing different levels of nisin and washed once with fresh M17 medium lacking nisin. The cultures were subsequently subcultured for 15 h in fresh medium with or without nisin (0.01–1 μg/liter).

DNA Techniques—General molecular techniques were performed as described by Sambrook *et al.* (19). Chromosomal and plasmid DNAs were isolated from *L. lactis* according to Johansen and Kibenich (20) and Birnboim (21), respectively. *L. lactis* was transformed with plasmid DNA by electroporation as described by Holo and Nes (22). All DNA modification enzymes were purchased from Roche Applied Science (Mannheim, Germany). PCRs were performed using Expand DNA polymerase and purified with a Roche PCR purification kit. Primers (listed in supplemental Table 1SM) were purchased from Biolegio BV (Malden, The Netherlands).

Construction of Strains and Plasmids—The genes *pgmH* and *galE* were cloned and overexpressed in *L. lactis* strain NZ9000 as follows. The coding regions of *pgmH* and *galE* were amplified by PCR using primer pairs yfgH-fw/yfgH-rev and galE-fw/galE-rev. The 0.76-kb RcaI/XbaI and 0.98-kb RcaI/SpeI fragments were digested with the indicated enzymes and cloned into NcoI/XbaI- or NcoI/SpeI-digested pNZ8048 (17), yielding constructs pNZ8048-*pgmH* and pNZ8048-*galE*, respectively. The *femD* gene was amplified using primer pair femD-fw/femD-rev. The pNZ8048-*femD* overexpression plasmid was constructed by cloning the 1.36-kb NcoI/XbaI PCR product into similarly digested pNZ8048. The resulting construct was transformed into *L. lactis* strain NZ9000. *L. lactis* MG1363 DNA (23) was used as template for all PCRs.

Several strategies were employed to construct an *L. lactis* Δ*pgmH* strain. A complete deletion of the *pgmH* gene was tried by using a two-step homologous recombination method as follows. The upstream and downstream flanking regions of *pgmH* were obtained by PCR using primer pairs yfgH-KO1/yfgH-KO2 and yfgH-KO3/yfgH-KO4 and cloned as EcoRI/BamHI and BamHI/XbaI restriction fragments into pORI280 (24), resulting in plasmid pORI280Δ*pgmH*. The plasmid was obtained and maintained in *L. lactis* LL302 (25). pORI280Δ*pgmH* and pVE6007 (26) were cotransformed into NZ9000, and this strain was taken through the temperature shift protocol for single and double crossovers (24). No double crossover transformants were obtained in M17 medium supplemented with following sugars at 0.5%: glucose, maltose, and trehalose or a mixture of trehalose (0.5%) and galactose (0.05%).

In a second approach, a 0.46-bp fragment containing the 5′-end of *pgmH* except for 2 bp was amplified by PCR using primers yfgH-fw1 and yfgH-rev1, double-digested with EcoRI/XbaI, and cloned into similarly digested pORI13 (27). The resulting plasmid was transformed into NZ9000, but despite several attempts, no erythromycin-resistant colonies were obtained.

Subsequently, pNZ8048-*pgmH* was introduced in NZ9000::pORI280Δ*pgmH*, obtained as explained above. The resulting chloramphenicol- and erythromycin-resistant strain was subjected to an excision strategy (24) in M17 medium containing glucose and nisin (0.1 μg/liter), yielding NZ9000Δ*pgmH*(*pgmH*$^+$). The addition of nisin was required to induce expression of *pgmH* from pNZ8048-*pgmH*.

Integration of plasmids in the chromosome and deletions were confirmed by PCR and Southern blotting. Probe labeling,

hybridization, and detection were performed using the ECL direct nucleic acid labeling system (Amersham Biosciences, Little Chalfont, UK).

Enzyme Assays—Enzymes were assayed at 30 °C after disruption of a cell suspension by passage through a French press (twice at 120 megapascals). Protein concentration was determined by the method of Bradford (28). Routinely and during enzyme purification, α- and β-PGM activities were assayed as described by Qian *et al.* (9). Reactions were started by the addition of 1.5 mM α-Glc-1-P or β-Glc-1-P, respectively.

Purification of Native α-PGM from L. lactis—All steps were performed in the presence of 0.5 mM EDTA and 5 mM 2-mercaptoethanol at 4 °C. Cell extracts were prepared from 135 g (wet weight) of galactose-grown MG1363 cells that were suspended in 50 mM triethanolamine (TEA) buffer (pH 7.2) containing 5 mM $MgCl_2$. Precipitation steps with protamine sulfate (0.25%, w/v) and solid ammonium sulfate were performed as described by Qian *et al.* (9). The precipitate in the range of 45–85% $(NH_4)_2SO_4$ saturation was dissolved in 50 mM TEA buffer (pH 7.2) containing 30 mM KCl and dialyzed against the same buffer. The sample was applied to a Superdex 200 gel filtration column in 20 mM BisTris propane (pH 6.9) containing 45 mM KCl, and α-PGM activity was detected in the flow-through fractions. Active samples were loaded onto a Resource Q column in the same buffer, and elution was carried out with a linear gradient of KCl (45–500 mM). Active fractions (which eluted at ~240 mM KCl) were dialyzed against 10 mM Tris-HCl (pH 7.3) containing 4 mM $MgCl_2$ and 1.6 M $(NH_4)_2SO_4$. The sample was applied to a phenyl-Sepharose column, and elution was carried out with a linear gradient of $(NH_4)_2SO_4$ (1.6 to 0 M). The positive fractions were dialyzed against 50 mM TEA buffer (pH 7.5) and 30 mM KCl and loaded onto a Superose 6 gel filtration column. α-PGM activity was measured in the flow-through fractions. Two putative target proteins of 28 and 37 kDa were excised from Coomassie Blue-stained SDS-polyacrylamide gels, and the amino acid sequences of their N termini were determined (29). Open reading frames encoding the two proteins were identified by BLASTp searches using the genome sequence of *L. lactis* ssp. *lactis* IL1403.

Purification and Characterization of Recombinant α-PGM—The protein was purified to electrophoretic homogeneity from ~65 g (wet weight) of glucose-grown nisin-induced NZ9000(*pgmH*$^+$) cells. A purification strategy similar to that used for the native enzyme was followed, except that the Superdex 200 gel filtration column was replaced with an anion-exchange Q-Sepharose column in 20 mM BisTris propane (pH 6.9) with a 45–500 mM KCl gradient, and the last chromatographic step (gel filtration) was not required. Also, the hydrophobic step (phenyl-Sepharose) preceded the anion-exchange step (Resource Q). The purified protein was stored at −20 °C in TEA buffer.

The pH profile was determined in TEA buffer at a range of 4–9. The effect of alternative cations (Ni^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, and Li^+) at 1 or 5 mM was examined in the presence of 50 μM Mg^{2+}. ATP and fructose 1,6-bisphosphate (Fru-1,6-P_2) were examined as potential inhibitors of α-PGM. Kinetic constants were determined in the reaction direction α-Glc-1-P → Glc-6-P. The V_{max} for the direction Glc-6-P → α-Glc-1-P and substrate specificity were determined by ^{31}P NMR spectroscopy. The 3-ml reaction mixtures contained 50 mM TEA buffer (pH 7.2), 5 mM $MgCl_2$, 50 μM Glc-1,6-P_2, 3% (v/v) ^2H_2O, and 20 μg of pure enzyme. For substrate specificity, spectra were acquired before and after 3 h of incubation at 30 °C in the presence of the following compounds at 7.5 mM: α-Glc-1-P, Glc-6-P, β-Glc-1-P, GlcN-1-P, GlcN-6-P, α-Man-1-P, Fru-1-P, Fru-6-P, Fru-1,6-P_2, Fru-1,6-P_2/α-Glc-1-P, α-Gal-1-P, Gal-6-P, Rib-5-P, ribulose-5-P, 6-phosphogluconate, UDP-Gal, and UDP-Glc. For V_{max} determinations, the reactions were started by the addition of Glc-6-P (50 mM), and the time course for its consumption was monitored. Methyl phosphonate was added as an internal concentration standard. Molecular mass was determined by gel filtration on a Superose 12 10/300 GL column using 100 mM sodium acetate (pH 7.0).

Determination of Extra- and Intracellular Metabolites during Growth—Samples (2 ml) of NZ9000(pNZ8048) or NZ9000(*pgmH*$^+$) cultures growing in chemically defined medium containing either glucose or galactose were collected at different points during growth. Fermentation substrates and products were quantified as described (30).

Ethanol extracts for analysis by ^{31}P NMR and quantification of phosphorylated metabolites in NZ9000(*pgmH*$^+$) and control strains at mid-exponential growth phase were prepared as described (31). Assignment of resonances and quantification of phosphorylated metabolites were based on previous studies (31) or determined by adding the suspected, pure compounds to the NMR sample extracts. The values reported for intracellular phosphorylated compounds are the averages of two independent growth experiments, and the accuracy was ~15%.

NMR Experiments and Quantification of Metabolites—Cells were harvested during the mid-logarithmic growth phase (A_{600} = 2.2), centrifuged, washed twice, and resuspended to a protein concentration of 16.5 mg/ml in 50 mM potassium P_i buffer (pH 6.5). *In vivo* NMR experiments were performed as described (18). Spectra were acquired sequentially prior to and after the addition of [1-^{13}C]glucose or [1-^{13}C]galactose. After substrate exhaustion and when no changes in the resonances due to end products and intracellular metabolites were observed, a total NMR sample extract was prepared and used for quantification of end products and other metabolites (32–34). Correction factors to convert peak intensities into concentrations for Gal-1-P (0.73), α-Glc-1-P (0.73), UDP-Gal (0.67), and UDP-Glc (0.67) were determined using NMR sample extracts supplemented with the pure compounds as described (32).

NMR Spectroscopy—NMR spectra of living cells were run at 30 °C as described by Neves *et al.* (18). Although individual experiments are depicted in the figures, each experiment was repeated at least twice, and the results were highly reproducible. The concentration values are the means of two to four experiments, and the accuracy varied from ±2% in the case of extracellular products to ±10% in the case of intracellular metabolites with concentrations below 5 mM. The quantification of phosphorylated metabolites and the measurement of α-PGM activity were performed as described by Ramos *et al.* (31). Carbon and phosphorus chemical shifts are referenced to external methanol or H_3PO_4 (85%) designated at 49.3 and 0.0 ppm, respectively.

Chemicals—[1-^{13}C]Glucose (99% enrichment) and [1-^{13}C]-galactose (99% enrichment) were obtained from Campro Scientific (Veenendaal, The Netherlands) and Cambridge Isotope Laboratories (Andover, MA), respectively. All other chemicals were reagent grade and obtained from Sigma.

RESULTS

Purification of α-PGM Activity and Identification of the Coding Gene

A BLASTp search of *L. lactis* MG1363 and IL1403 genomes using sequences of proteins in the α-D-phosphohexomutase superfamily identified *femD* as the best hit. FemD, annotated as a putative phosphoglucosamine mutase (14), is a protein with a calculated molecular mass of 48 kDa. To ascertain whether *femD* encodes a protein with α-PGM activity, the gene (1356 bp) was cloned into pNZ8048 under the control of the nisin-inducible promoter and introduced into NZ9000. Extracts of nisin-induced NZ9000(pNZ8048-*femD*) cells contained a clearly overproduced protein with a size of 48 kDa (data not shown). Moreover, the activity of α-PGM in the induced strain was identical to that in control cells (0.15 units/mg of protein), indicating that *femD* does not encode an α-PGM. A PCR strategy to clone the lactococcal α-PGM gene using degenerate primers based on highly conserved regions of phosphohexomutases (16) and the complementation of an *Escherichia coli pgm*::*tet* mutant (35) with a genomic library of *L. lactis* were also attempted without success. Consequently, purification of α-PGM activity was pursued to obtain protein sequence information and to identify the gene.

α-PGM was partially purified (100-fold). Fractions from the last column contained several bands as visualized by SDS-PAGE, but only two proteins (28 and 37 kDa) co-eluted with the PGM activity in all purification steps. These two protein bands were excised, and their N-terminal amino acid sequences were determined to be MKKILSFD and MTVLVLG, respectively. The corresponding open reading frames in the *L. lactis* IL1403 genome sequence were *yfgH* (NP_266726, hypothetical protein) and *galE* (NP_268136, UDP-glucose 4-epimerase), respectively. The product of *galE* was shown to be UDP-glucose 4-epimerase by others (36, 37), and cloning and overexpression of *galE* in NZ9000 did not lead to enhancement of α-PGM activity (data not shown). The *yfgH* (herein renamed *pgmH*) gene contains 759 bp and codes for a protein with 252 amino acids and a calculated molecular mass of 28,276 kDa. The gene from MG1363 was cloned into pNZ8048 and expressed in strain NZ9000. Induction with nisin of an NZ9000(*pgmH*$^+$) culture grown on Glc-containing M17 medium resulted in a 30-fold increase in α-PGM activity, thus proving that *pgmH* encodes *L. lactis* α-PGM.

In the chromosome of *L. lactis* MG1363, *pgmH* is flanked by *yfgL* and *yfgG* (Fig. 1*A*), both of unknown function.[4] All three genes are preceded by Shine-Dalgarno sequences (5′-AAATAGGAGA-3′ for *pgmH*). A putative promoter region containing an extended −10 sequence (5′-TGTTATAAT-3′) precedes the *pgmH* coding sequence. Inverted repeat sequences (5′-AAAAAGCAATCTATTTTGATTAGATTGTTTTT-3′ and 5′-AAAAAGTTGTCATTAATGACAGCTTTTT-3′) followed by AT-rich regions downstream of the *yfgL* and *pgmH* stop codons, respectively, could function as transcriptional terminators. Therefore, it is unlikely that the three genes are organized in an operon-like structure, and possibly, they have unrelated functions. A similar genomic organization has been observed for *L. lactis* ssp. *lactis* IL1403 (14).

Interestingly, the product of *pgmH* (DQ778336) (Fig. 1*B*) has no sequence homology to proteins from the α-D-phosphohexomutase family. It shows ~37% identity to hypothetical proteins from human colonizing Gram-positive bacteria (ZP_00121741, *Bifidobacterium longum*; and YP_056695, *Propionibacterium acnes*) and ~25% identity to eukaryotic phosphomannomutases (PMMs) (Fig. 1*B*). These proteins have been classified as members of the HAD superfamily (PF03332; www.sanger.ac.uk/cgi-bin/Pfam). Despite the low overall identity, the α-PGM sequence contains the four conserved motifs that characterize the HAD superfamily: motif I (**D***X***D***X*(T/V)), motif II ((**S**/T)*XX*), and motifs III and IV (**K**X_{18-30}(**G**/S)(**D**/S)) (38, 39).

Biochemical Characterization of Recombinant α-PGM

The protein was purified ~13-fold to a specific activity of 65 units/mg of protein. The biochemical and kinetic properties are listed in Table 2. The purified protein was electrophoretically homogeneous, and SDS-PAGE revealed a single subunit with an apparent molecular mass of 28 kDa. A mass of 84.3 kDa was determined by gel filtration (Superose 12 10/300 GL), suggesting a trimeric structure. The pH for maximal activity was 6.5; 50% of the activity was found at pH 5.6 and 7.5. The rate dependence on α-Glc-1-P concentration followed Michaelis-Menten kinetics. Several phosphosugars were examined as putative substrates using the ^{31}P NMR direct assay. The enzyme catalyzed only the interconversion of α-Glc-1-P to Glc-6-P. Furthermore, the apparent V_{max} value for the reverse direction (Glc-6-P → α-Glc-1-P) was 3-fold lower than that for the forward direction, and the apparent K_m value for Glc-6-P was in the millimolar range. Neither α-Man-1-P nor β-Glc-1-P was a substrate for the enzyme, despite the sequence similarity of *L. lactis* α-PGM to the eukaryotic PMMs (~25%) and β-PGM to Glc-1-P phosphodismutases (~10%).

For maximal activity, α-PGM required Mg^{2+} and Glc-1,6-P_2. In the absence of Mg^{2+}, the activity was reduced to <2%, and no activity was detected when Glc-1,6-P_2 was omitted from the assay. Zn^{2+} and Ca^{2+} strongly inhibited the activity (Table 2). ATP showed no regulatory effect, whereas Fru-1,6-P_2 moderately inhibited the activity.

Pools of Glycolytic Intermediates in Non-growing Cells

L. lactis NZ9000 cells harboring pNZ8048-*pgmH* or pNZ8048 (control) were grown in a chemically defined medium containing glucose or galactose and induced with nisin for α-PGM production. The effect of α-PGM overproduction on the metabolism of glucose and galactose was studied by *in vivo* ^{13}C NMR (Fig. 2).

[1-^{13}C]Glucose—The conversion of glucose was homofermentative in the NZ9000(*pgmH*$^+$) and NZ9000(pNZ8048) strains (lactate production above 91%), with the glucose consumption rates being 0.37 ± 0.02 and 0.39 ± 0.02 μmol/min/mg of protein. The dynamics of intracellular metabolite

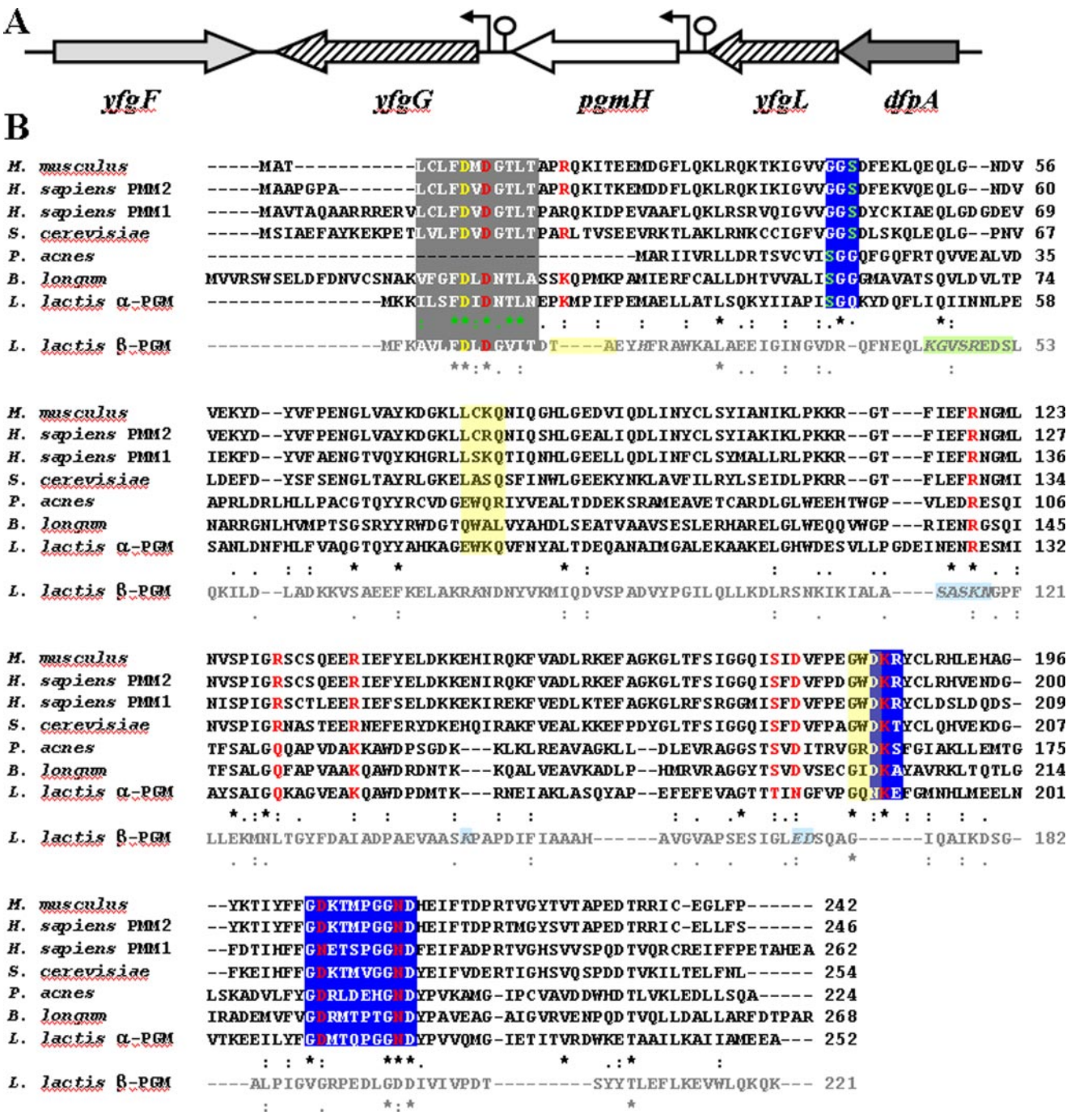

FIGURE 1. **Multiple sequence alignment of amino acid sequences of α-PGM from *L. lactis* ssp. *cremoris* MG1363 and its putative homologs from *P. acnes*, *B. longum*, *Mus musculus*, *Homo sapiens* (PMM1 and PMM2), and *Saccharomyces cerevisiae*.** The alignment was generated with ClustalW. The eukaryotic α-PMMs belong to subfamily II of the HAD superfamily. Lactococcal β-PGM, a subfamily I member, is shown in *gray*. The signature pattern for motif I of the HAD superfamily phosphomutases, -D*X*D*X*T- (metal binding and nucleophile), is highlighted by the *dark gray box* (42). The invariant aspartate residue is shown in *yellow*. The other signature patterns of HAD superfamily subfamily II (motifs II–IV) are highlighted by the *blue dark boxes* (39, 47). The *light blue* and *light green boxes* highlight core motifs 1–4 and cap loop 5, respectively, of β-PGM (43). The *yellow boxes* indicate hinge regions connecting core and cap domains. Residues proposed to be important for substrate binding or catalysis are shown in *red*. The conserved serine residue in motif II is shown in *green* (residue inversion in α-PMM and lactococcal α-PGM and its homologs). For accession numbers, see "Results."

pools were not appreciably affected by the substantial increase in α-PGM activity from 0.07 units/mg of protein in the control to 2.6 units/mg of protein in NZ9000(*pgmH*$^+$) (Fig. 2, *A* and *B*). The maximal concentration of Fru-1,6-P_2 decreased by ~10%, and 3-phosphoglycerate increased slightly. Moreover, the UDP-glucose pool decreased from 6.7 ± 0.5 mM in the control to 4.2 ± 0.3 mM in NZ9000(*pgmH*$^+$) (Fig. 2, *A* and *B*).

[1-^{13}C]Galactose—As expected for galactose, the metabolism of pyruvate was shifted to products other than lactate, ethanol (2.2 ± 0.2 mM), and acetate (2.4 ± 0.4 mM), but their concentrations were ~35% lower in NZ9000(*pgmH*$^+$). Induction with nisin resulted in a 7-fold higher (0.3–2.1 units/mg of protein) α-PGM activity in the strain harboring the *pgmH* construct, a modest change compared with the 37-fold increase

TABLE 2
Biochemical and kinetic properties of α-PGM from *L. lactis*

Parameter	Value
Enzyme apparent molecular mass (kDa)	
Native	84.3
Subunit	28
Calculated	28.3
Oligomeric structure	α3
pH optimum	6.5 ± 0.5
K_m for α-Glc-1-P (μM)	71.4 ± 2.8
K_{act} (μM)	
Glc-1,6-P_2	16.8 ± 1.3
Mg^{2+}	52.6 ± 5.1
V_{max} (units/mg protein)	
α-Glc-1-P	65.3 ± 1.8
Glc-6-P	15.8 ± 1.3
Substrate specificity (%)	
α-Glc-1-P (7.5 mM)	100[a]
Glc-6-P (50 mM)	23
β-Glc-1-P (7.5 mM)	0[b]
Effect of cations (%)	
Mg^{2+} (0 and 0.05 mM)	<2[c] and 49
Zn^{2+} (5 and 1 mM)	2 and 28
Ca^{2+} (5 and 1 mM)	0.3 and 1.6
Mn^{2+} (5 and 1 mM)	17 and 21
Ni^{2+} (5 and 1 mM)	43 and 41
Li^{+} (5 and 1 mM)	42 and 39
Potential inhibitors (I_{50}; mM)	
Fru-1,6-P_2	48 ± 1.1
ATP	No effect

[a] Activities are relative to the value determined for the conversion of α-Glc-1-P to Glc-6-P (which was set to 100%) as measured by ^{31}P NMR.
[b] The following phosphosugars were also examined, but no activity was detected: GlcN-1-P, GlcN-6-P, α-Man-1-P, Fru-1-P, Fru-6-P, Fru-1,6-P_2, α-Gal-1-P, Gal-6-P, Rib-5-P, ribulose-5-P, 6-phosphogluconate, UDP-Gal, and UDP-Glc. When a mixture of Fru-1,6-P_2 and α-Glc-1-P was used, α-Glc-1-P was fully converted to Glc-6-P, but Fru-1,6-P_2 was not used.
[c] Activities are relative to the value determined for the conversion of α-Glc-1-P to Glc-6-P (which was set to 100%) as measured using the standard coupling assay and 5 mM Mg^{2+}.

observed in glucose-grown cells. Curiously, the galactose consumption rate was 25% greater (from 0.16 ± 0.1 to 0.21 ± 0.1 μmol/min/mg of protein) in the strain overproducing α-PGM. Overproduction of α-PGM had a considerable impact on the concentrations of intracellular metabolite pools (Fig. 2, *C* and *D*). A remarkable reduction in the size of the Gal-1-P and α-Glc-1-P pools to 2.4 and 2.2 mM, respectively, revealed α-PGM as the main bottleneck during galactose metabolism in *L. lactis*. UDP-Glc (3.9 ± 0.4 mM) and UDP-Gal (3.2 ± 0.3 mM) were detected, and these pools were 1.4-fold lower in the strain with increased α-PGM activity (Fig. 2, *C* and *D*). As in the control strain, the accumulation of Fru-1,6-P_2 was slightly delayed, but in NZ9000(*pgmH*$^+$), its maximal concentration was clearly higher. The observations in resting cells raised the question as to how a growing culture would respond to an increase in α-PGM activity. Therefore, we investigated the effect of overexpression of *pgmH* in growing cells.

Impact of α-PGM Overproduction in Growing Cells

Growth Characteristics—The effect of overproduction of α-PGM on the growth properties of *L. lactis* was evaluated using either glucose or galactose as the carbon source (Table 3; see also supplemental Fig. 1SM). The specific activity of α-PGM measured in cells before the addition of nisin was consistently higher in the strain carrying pNZ8048-*pgmH*, most likely due to low basal P_{nisA} expression. Galactose *per se* induced α-PGM activity 2- and 4-fold in NZ9000(*pgmH*$^+$) and the control strain, respectively (Table 3). Up-regulation of *pgmH* expression on galactose was also observed for the parent strains MG1363 and NZ9000.

The growth rate was affected by nisin addition, but the magnitude and sign of the effect were sugar-dependent (Table 3). The growth rate on glucose decreased considerably upon the addition of nisin, but this negative effect was unrelated to α-PGM overproduction (16-fold increase). Despite the lower α-PGM activity achieved on galactose (7-fold increase), the growth rate of NZ9000(*pgmH*$^+$) was substantially greater than that of the control strain (0.50 *versus* 0.36 h^{-1}), reaching a value close to that on glucose (0.56 h^{-1}). The results show that α-PGM activity in the control strain was limiting during growth on galactose and that this bottleneck was overcome by *pgmH* overexpression.

In glucose-grown cells, increased α-PGM activity had no impact on product formation, with lactate accounting for >90% of the end products. When galactose was used as the carbon source, the lactate yield increased slightly in the strain overproducing α-PGM (Fig. 3 and Table 3), in line with the increased growth rate of NZ9000(*pgmH*$^+$). Biomass yield, ATP yield, and biomass yield on ATP were dependent on the sugar used and were not affected by overproduction of α-PGM (Table 3).

Pools of Phosphorylated Metabolites—Pool sizes for glycolytic intermediates and sugar nucleotides were determined by ^{31}P NMR in cell extracts derived from mid-exponential phase cultures (supplemental Table SM2). Fru-1,6-P_2 was the major metabolite on glucose, whereas galactose 3-phosphoglycerate and phosphoenolpyruvate were also present in high amounts. As expected, the Leloir pathway intermediate Gal-1-P was below the detection limit on glucose; on galactose, its concentration was slightly lower (20%) in the α-PGM-overproducing strain. The concentration of α-Glc-1-P decreased notably in NZ9000(*pgmH*$^+$) regardless of the sugar used.

It is thought that, in *L. lactis*, UDP-sugars and UDP-aminosugars are derived from α-Glc-1-P and Fru-6-P, respectively; hence, their concentrations could respond to changes in α-PGM activity. Overproduction of α-PGM resulted in reduction or constancy of UDP-sugars on galactose- or glucose-grown cells, respectively. Concentrations of UDP-aminosugars were not significantly affected by *pgmH* overexpression, except for UDP-*N*-acetylglucosamine, the first cytoplasmic precursor of peptidoglycan, the level of which responded inversely to an increase in the activity of α-PGM.

Inactivation of the Chromosomal pgmH Gene and Its Effect on L. lactis Growth

To investigate whether α-PGM activity is essential for growth of *L. lactis* on glucose and galactose, we decided to inactivate the *pgmH* gene. Several attempts to disrupt *pgmH* by single crossover plasmid integration with pORI13-*pgmH*′ or by a method to introduce unmarked deletions failed. These results suggest that *pgmH* plays an essential role in *L. lactis*. Integration of pORI280Δ*pgmH* in NZ9000 resulted in erythromycin-resistant colonies harboring a disrupted as well as an integral copy of *pgmH*. Only when *pgmH* was expressed in *trans* (under nisin

control in pNZ8048) in NZ9000::pORI280Δ*pgmH* was it possible to delete the chromosomal copy of *pgmH*, as confirmed by PCR and Southern analysis (data not shown). The resulting strain, NZ9000Δ*pgmH*(*pgmH*$^+$), was constructed and maintained in the presence of 0.1 μg/liter nisin. To ascertain whether α-PGM was limiting during growth on Glc-containing M17 medium, low nisin (0.01 μg/liter)-grown cultures (α-PGM activity of 0.11 units/mg of protein) were subcultured in fresh medium with increasing concentrations of nisin (0–1 μg/liter). In the absence of nisin, the mutant strain showed poor growth, with an average growth constant of 0.20 h^{-1}; at 12 h, α-PGM activity had decreased to 0.025 units/mg of protein. Under the same conditions, the NZ9000(pNZ8048) control strain showed a growth rate of 0.55 h^{-1} and reached a 5-fold higher biomass concentration (A_{600} = 3.2 compared with 0.6) and a steady α-PGM activity of 0.15 units/mg of protein. Modulation of *pgmH* expression by varying the nisin concentration from 0.01 to 1 μg/liter resulted in a series of cultures with α-PGM activity between 63 and 4000% of the control level (supplemental Fig. 2SM). The maximal growth rate was negatively affected at the lowest α-PGM activity (63% of the control). The data show that α-PGM activities below the control level did not sustain maximal growth of *L. lactis* on Glc-containing M17 medium.

The effect of controlled limitation of *pgmH* expression was evaluated during growth on glucose and galactose in M17 medium. Strain NZ9000Δ*pgmH*(*pgmH*$^+$) was grown in medium containing different concentrations of nisin and subsequently subcultured in nisin-free medium. In Glc-grown cells, nisin concentrations of 0.1, 0.01, and 0 μg/liter in the inocula resulted in 16, 65, and 74% reductions in the growth rates, respectively. In contrast, nisin concentrations of 0.5 and 1 μg/liter supported growth constants similar to those of the control strain. The effect on growth rates, as well as the stepwise reduction in the final absorbances, correlated well with the decline of α-PGM activity (supplemental Fig. 2SM). These results show that *pgmH* is essential for growth of *L. lactis* on glucose.

When galactose was used as the sole carbon source, the effect of limiting the expression of *pgmH* on the growth rates and final absorbance was more pronounced than with glucose. Regardless of the nisin concentration used (0–1 μg/liter), the

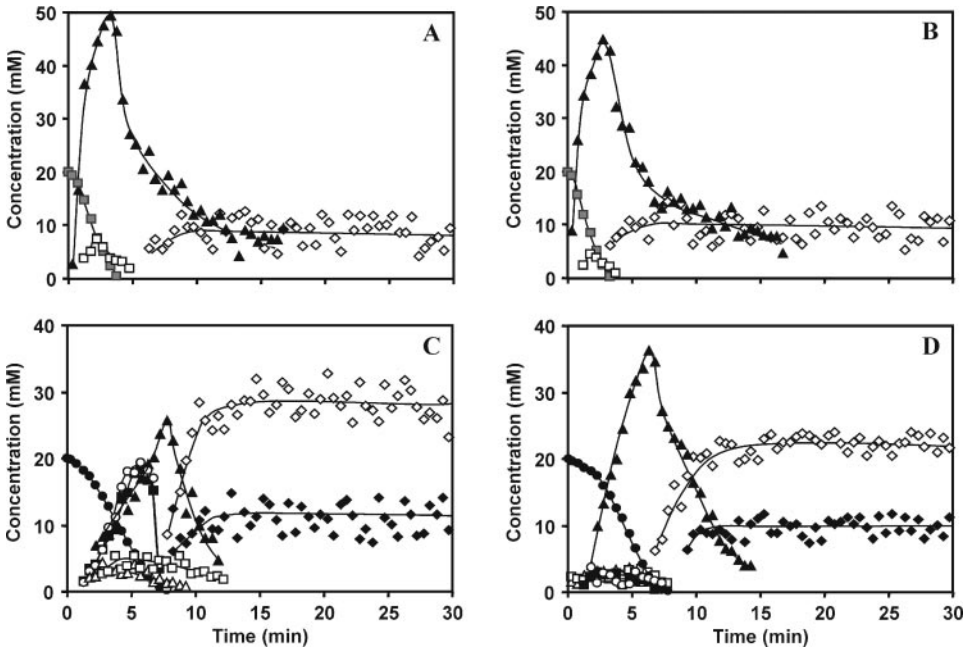

FIGURE 2. **Metabolism of glucose or galactose in non-growing cell suspensions of *L. lactis* NZ9000(pNZ8048) and NZ9000(*pgmH*$^+$).** Shown is the time course for substrate consumption and pools of intracellular metabolites in non-growing cultures of strains NZ9000(pNZ8048) (*A* and *C*) and NZ9000(*pgmH*$^+$) (*B* and *D*) during the metabolism of [1-^{13}C]glucose (*A* and *B*) and [1-^{13}C]galactose (*C* and *D*) as monitored by ^{13}C NMR. Cells were grown in a bioreactor vessel on glucose (*A* and *B*) or galactose (*C* and *D*) in deaerated chemically defined medium at pH 6.5 and induced with nisin (1 μg/liter) at A_{600} = 0.5. Lactate, ethanol, and 2,3-butanediol were also detected, but their time courses were omitted from the graphs for the sake of simplicity. *Gray square*, glucose; ●, galactose; ▲, Fru-1,6-P_2; ■, α-Glc-1-P; ○, Gal-1-P; ◇, 3-phosphoglycerate; ◆, phosphoenolpyruvate; □, UDP-Glc; △, UDP-Gal. The fitted *lines* are simple interpolations.

TABLE 3

Effect of *pgmH* overexpression on some growth properties during glucose or galactose fermentation by *L. lactis*

NZ9000(*pgmH*$^+$) and NZ9000(pNZ8048) were grown in chemically defined medium supplemented with chloramphenicol (5 mg/liter) and 1% (w/v) glucose or galactose. Nisin (1 μg/liter) was added when the culture reached A_{600} = 0.5. Growth rate constants for the entire growth phase were calculated using linear regressions. α-PGM activity was measured before (A_{600} = 0.5) and after (A_{600} = 2.2) induction. Y_{ATP} is the biomass yield relative to ATP production. The global ATP yields were calculated from the fermentation products assuming that all ATP was synthesized by substrate level phosphorylation.

	Glucose		Galactose	
	pNZ8048	*pgmH*$^+$	pNZ8048	*pgmH*$^+$
Carbon balance (%)	95 ± 0.5	95 ± 0.5	94 ± 0.3	94 ± 0.1
Biomass yield (g/mol)	29.9 ± 0.3	29.1 ± 0.3	26.5 ± 0.2	26.5 ± 0.4
ATP yield (mol/mol substrate)	1.9 ± 0.01	1.9 ± 0.01	2.1 ± 0.01	2.1 ± 0.01
Y_{ATP}	15.6 ± 0.02	15.2 ± 0.02	12.5 ± 0.02	12.9 ± 0.02
μ_1[a]	0.80 ± 0.01	0.82 ± 0.01	0.36 ± 0.01	0.45 ± 0.01
μ_2[b]	0.58 ± 0.01	0.56 ± 0.02	0.37 ± 0.01	0.50 ± 0.01
α-PGM activity (units/mg)[a]	0.07 ± 0.01	0.16 ± 0.01	0.29 ± 0.01	0.36 ± 0.02
α-PGM activity (units/mg)[b]	0.07 ± 0.01	2.63 ± 0.20	0.34 ± 0.02	2.05 ± 0.16
Lactate/substrate (%)	92 ± 0.4	91 ± 0.4	71 ± 1.0	79 ± 0.8
Other products/substrate (%)	2 ± 0.5	3 ± 0.7	23 ± 0.3	16 ± 0.3

[a] Before the addition of nisin.
[b] After the addition of nisin (1 μg/liter).

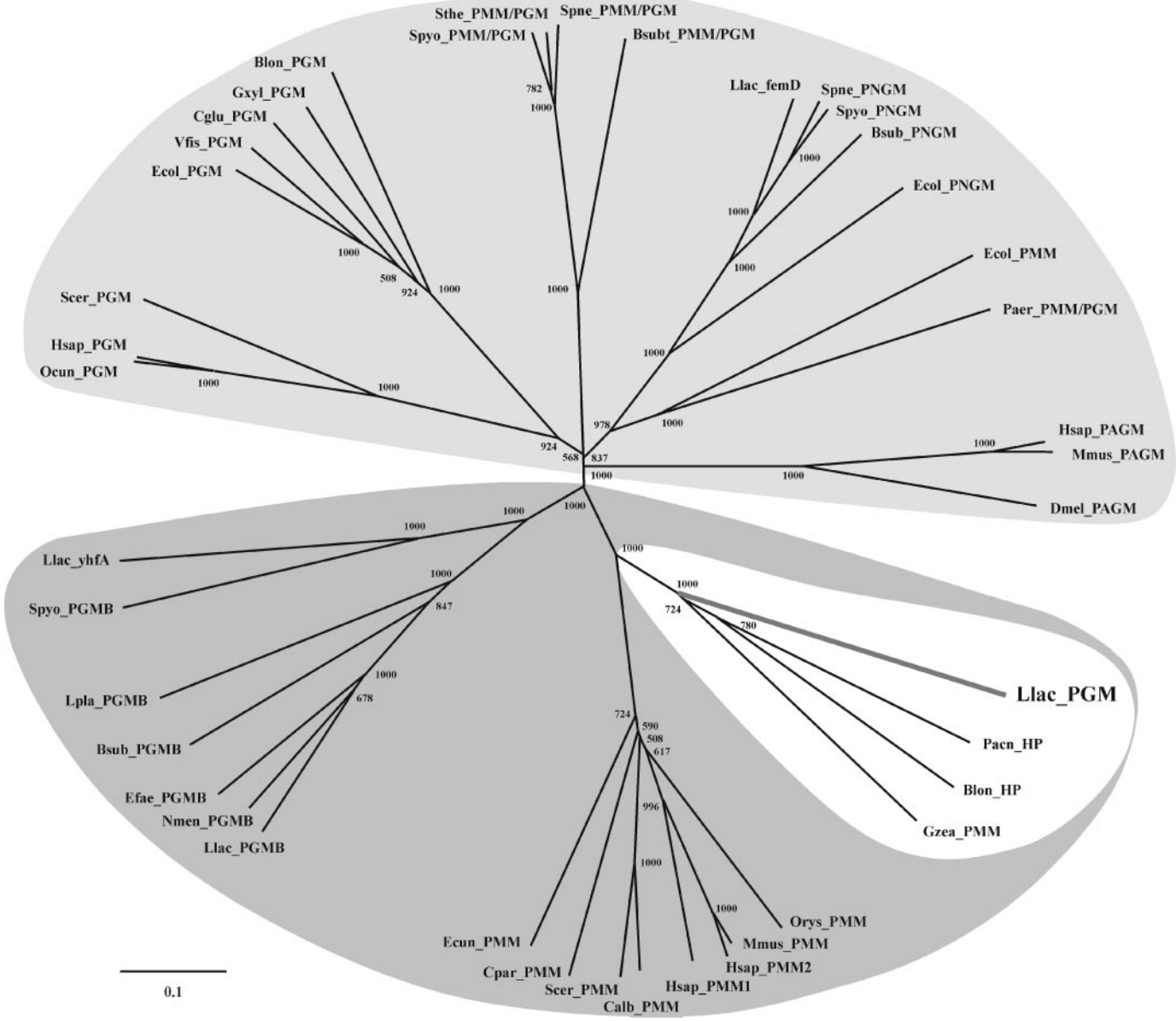

FIGURE 3. **Unrooted phylogenetic tree based on available amino acid sequences of phosphomutases.** The ClustalX program (48) was used for sequence alignments and to generate the phylogenetic tree with the neighbor-joining tree construction method. The significance of the branching order was evaluated by bootstrap analysis of 1000 computer-generated trees. The bootstrap values are indicated. *Bar* = 0.1 change/site. The species and GenBank™ accession numbers are as follows: *Oryctolagus cuniculus* (*Ocun*) PGM (P00949); *S. cerevisiae* (*Scer*) PGM (NP_013823) and PMM (NP_116609); *H. sapiens* (*Hsap*) PGM (AAH67763), phosphoacetylglucosamine mutase (*PAGM*; AAD55097), PMM1 (AAC51117), and PMM2 (AAH08310); *L. lactis* (*Llac*) FemD (NP_266580), α-PGM (*pgmH*; DQ778336), β-PGM (*PGMB*; NP_266585), and YhfA (NP_266899); *Streptococcus pneumoniae* (*Spne*) phosphoglucosamine mutase (*PNGM*; AAL00221) and PMM/PGM (AAD56627); *Streptococcus pyogenes* (*Spyo*) phosphoglucosamine mutase (YP_280210), PMM/PGM (YP_282301), and β-PGM (YP_281891); *Bacillus subtilis* (*Bsub*) phosphoglucosamine mutase (NP_388058), PMM/PGM (CAH04980), and β-PGM (NP_391335); *E. coli* (*Ecol*) phosphoglucosamine mutase (AAC76208), PMM (O85343), and PGM (AAC73782); *M. musculus* (*Mmusc*) phosphoacetylglucosamine mutase (NP_082628) and PMM (NP_058577); *Drosophila melanogaster* (*Dmel*) phosphoacetylglucosamine mutase (NP_648588); *P. acnes* (*Pacn*) hypothetical protein (*HP*; YP_056695); *B. longum* (*Blon*) hypothetical protein (ZP_00121741) and PGM (NP_696782); *G. zeae* (*Gzea*) PMM (EAA71459); *Oryza sativa* (*Orys*) PMM (XP_474395); *Cryptosporidium parvum* (*Cpar*) PMM (EAK87737); *Encephalitozoon cuniculi* (*Ecun*) PMM (CAD26542); *Candida albicans* (*Calb*) PMM (EAL02637); *Pseudomonas aeruginosa* (*Paer*) PMM/PGM (NP_254009); *Streptococcus thermophilus* (*Sthe*) PMM/PGM (AAV62380); *Gluconacetobacter xylinus* (*Gxyl*) PGM (P38569); *Corynebacterium glutamicum* (*Cglu*) PGM (CAF21203); *Vibrio fischeri* (*Vfis*) PGM (AAM77720); *Neisseria meningitides* (*Nmen*) β-PGM (CAB85309); *Enterococcus faecalis* (*Efae*) β-PGM (NP_814693); and *Lactobacillus plantarum* (*Lplan*) β-PGM (NP_783891). α-D-Phosphohexomutase and HAD superfamilies are highlighted by *light gray* and *dark gray areas*, respectively. The *white area* indicates the cluster in the HAD superfamily that includes *L. lactis* α-PGM.

final biomass and growth rate constants were below 60% of the respective parameters in the control strain (A_{600} = 3.5 and μ = 0.41 h^{-1}). An α-PGM activity of 1.38 units/mg of protein was measured at 12 h in cells grown and subcultured in fresh medium with nisin at 0.5 μg/liter, but the growth parameters did not improve. Although no final explanation for this intriguing behavior can be put forward, it is possible that complex regulatory mechanisms resulting from cross-reactions involving galactose metabolism and nisin induction are implicated (40, 41). The severe growth defects shown here for *L. lactis* NZ9000Δ*pgmH*(*pgmH*$^+$) validate the conclusion that *pgmH* is essential for growth of *L. lactis* on galactose.

DISCUSSION

In this study, we have described the identification and functional analysis of *L. lactis* α-PGM, the first characterized member of a novel α-PGM family.

Sequence Comparison and Properties—All α-PGMs characterized so far belong to the α-D-phosphohexomutase superfamily of proteins (16). Interestingly, the α-PGM encoded by *L. lactis pgmH* has no sequence homology to members of the α-D-phosphohexomutase superfamily and does not contain the family's consensus motifs. Instead, it shows sequence homology to eukaryotic PMMs (Figs. 1 and 3), an unrelated group of proteins that, despite their phosphohexomutase activity, belong to the HAD superfamily. The *pgmH* product features the conserved sequence motifs characteristic of the HAD superfamily (38, 39, 42, 43) and is similar in size to the eukaryotic PMMs (Fig. 1). The HAD superfamily comprises two branches that have acquired phosphohexomutase function, the eukaryotic α-PMMs (PF03332) and β-PGMs (PF00702) (11). As all known α-PGMs fall into the α-D-phosphohexomutase superfamily, we propose that the *L. lactis* α-PGM represents a novel line of α-D-phosphohexomutase evolution (Fig. 3).

Unlike the eukaryotic PMMs, which in general use both Man-1-P and Glc-1-P, *L. lactis* α-PGM shows strict specificity for α-Glc-1-P. Substrate specificity has been related to subtle residue variance in the catalytic domains (16). In the HAD superfamily, the catalytic cycle proceeds via a bisphosphorylated sugar intermediate to the reversible conversion of 1- to 6-phosphosugars and requires Mg^{2+} as a cofactor (Table 2) (43–45). Within this superfamily, the hexose C-1 configuration (α- or β-anomer) specificity and sequence/structural features allow us to distinguish between the eukaryotic α-PMMs and β-PGMs (Fig. 1) (12, 43, 46, 47). The position and fold of the cap domain (Fig. 1) place *L. lactis* α-PGM in HAD superfamily subclass II, whereas β-PGM is a subclass I protein. The sequence identities between *L. lactis* α-PGM (query sequence) and human α-PMM1 and *L. lactis* β-PGM are 25 and 10%, respectively. The structural and sequence similarities and the anomeric specificity suggest that *L. lactis* α-PGM is mechanistically closer to α-PMMs than to β-PGMs. This hypothesis is strengthened by the presence of the residues involved in the catalytic process of α-PMM1, in particular the nucleophile Asp^{8}, the acid/base Asp^{10}, and Gln^{51} (Asp^{19}, Asp^{21}, and Gln^{62} in human α-PMM1). However, only some of the conserved positively charged residues at the interface of the cap and core domains in α-PMMs are present in *L. lactis* α-PGM (Fig. 1), suggesting a mechanism of action different from that of the electrostatic wedge proposed for α-PMM (47).

A phylogram including both characterized and putative phosphohexomutases from eukaryotic and bacterial sources is given in Fig. 3 (48). The depicted topology clearly separates members of the α-D-phosphohexomutase and HAD superfamilies despite their similar function. The phylogenetic tree reflects the family division in the HAD superfamily, with bacterial β-PGMs and eukaryotic α-PMMs clustered into two distinct groups. *L. lactis* α-PGM is included in a cluster comprising proteins with unknown function of the human-associated organisms *B. longum* and *P. acnes* and the plant pathogen *Gibberella zeae*, which branches from the line leading to the eukaryotic α-PMMs. This suggests an origin common to these proteins and the eukaryotic α-PMMs, which also share the same α-anomeric specificity. A possible explanation relies on independent lateral gene transfer from eukaryotes to their commensal or pathogenic organisms. Surprisingly, of all the bacteria with available genome sequences, *L. lactis* appears to be unique insofar as it lacks an α-PGM of the α-D-phosphohexomutase superfamily.

Physiological Function of L. lactis α-PGM—In this work, we have shown that the highly specific α-PGM mediates the reversible conversion of α-Glc-1-P to Glc-6-P in *L. lactis*. Inactivation of α-PGMs is a trivial procedure in bacteria (6, 7, 49–55). Remarkably, all strategies attempted to disrupt *pgmH* failed. Our experiments with the *pgmH* conditional knock-out strain showed dramatic effects on growth rate and final biomass when the activity was below the control level. Although residual α-PGM activity could still sustain modest growth, cell division and morphology were affected as denoted by the appearance of long chains comprising cells that had lost their typical lactococcal shape (data not shown). *L. lactis* strains deficient in UDP-galactose 4-epimerase (*galE*) (56), the major autolysin (*acmA*) (57), or lipoteichoic acid D-alanylation (58) shows a similar behavior, suggesting altered or deficient biosynthesis of cell wall polysaccharides. Earlier attempts to inactivate *L. lactis* MG1363 *galU*, which encodes the enzyme that catalyzes the conversion of α-Glc-1-P to UDP-Glc, were also unsuccessful (56); and as for α-PGM, no other genes coding for a galactose uridyltransferase were found in the available genome sequences (14).[4] Therefore, it is reasonable to conclude that UDP-Glc synthesis in *L. lactis* relies entirely on *pgmH* and *galU* gene products.

Overproduction of α-PGM affected the levels of glycolytic metabolites and the glycolytic flux from galactose, but not from glucose, reflecting the different role of *pgmH* in the metabolism of these two sugars: α-PGM is required for galactose degradation and was identified as a bottleneck in galactose metabolism, whereas its main function is providing precursors for biosynthetic pathways during growth on glucose. Altogether, the results presented here support the conclusion that *pgmH* is the only gene in the *L. lactis* genome coding for α-PGM activity. Therefore, the purification of a 65-kDa protein with α-PGM activity reported previously (9) can only be explained on the basis of the different genetic backgrounds used.

This work revealed a novel α-D-PGM that falls into the HAD family, unlike all other known α-PGMs. The demonstration of the essentiality of *pgmH* is a final confirmation of the crucial role played by the enzyme in the physiology of *L. lactis*. To further understand the unique features unraveled by this study, the structural determination of this new α-D-PGM is in progress.

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